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SCIENTIFIC VALIDATION OF SIDDHA FORMULATION RASA PARPAM AND ITS ANTICANCER PROPERTY IN HELA CELL LINE- AN INVIVO AND INVITRO ASSAY

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ABSTRACT

Cancer is a large family of diseases caused by an uncontrolled division of abnormal cells in a part of the body. Cancer has become a topic of great scientific interest since its awareness to the medical world, because of its obscure etiological factors. Cancer is known as "Putru" in Siddha system which literally means "Termite mound" because of its proliferative nature. In India, tumors (neoplasms) are the third cause of mortality. Cervical cancer is the second most common cancer in the women worldwide and the leading cause of cancer deaths among women in developing countries.

KEYWORDS: Anti-cancer, HeLa cell line, cervical cancer, Rasaparpam.

INTRODUCTION

Cancer is characterized by never-ending and uncontrolled anarchic cell proliferation. This anarchic proliferation of abnormal *cells is* opposed to the controlled, harmonious proliferation of normal tissues which only occurs to repair damaged or worn tissues. Carcinogenic factors (tobacco, alcohol, pollutants, chemicals, radioactivity, vaccines, viruses, aging, stress, violence, despair, etc.) produce mutations or methylations. Cancer is the second leading cause of clinical mortality in developed countries. Cancer and tumors are the result of altered, excessive and invasive cell reproduction in other nearby healthy tissues, their development can be originated from diverse causes, like genetics, to the consumption of potentially toxic and harmful foods. In India, tumors (neoplasms) are the third cause of mortality. In this

category, malignant tumors of the cervix are the first cause of female death. Cervical cancer is the second most common cancer in the women worldwide and the leading cause of cancer deaths among women in developing countries.[1] Sexually transmitted human papilloma virus (HPV) infection is the most important risk factor for cervical intraepithelial neoplasm and invasive cervical cancer.[2] HPV serotypes 16 and 18 account for nearly 76.7% of cervical cancer in India. Warts have been reported in 2–25% of sexually transmitted disease clinic attendees in India; however, there is no data on the burden of anogenital warts in the general community.[3] Every year in India, 122,844 women are diagnosed with cervical cancer and 67,477 die from the disease.[4] HPV is a virus that can lead to conditions as harmless as a wart and as deadly as cervical cancer. Although there is no cure for HPV, there are preventative measures and herbs with antiviral properties that might be able to reduce the adverse effects and spread of the disease. Human papilloma virus (HPV) is a virus that can infect human mucous membranes and epidermis, which can lead to cancers of the vagina, anus, cervix, vulva and penis. There are more than 100 different types that are usually divided into those that cause warts, those that cause cancer, and those that have no symptoms at all (which are essentially harmless). Some types of viruses can be sexually treated (like the strain that causes genital warts) and some have a chance to get rid of on their own within two years of 90 percent. The Pap smear test is intended to detect the cells that can develop in the cervix of the uterus as a result of HPV. In Modern system of medicine, the main strategy of treating patients with cancer are surgery, radiation and chemotherapy.[5] Most patients unfortunately seek Siddha system of medicine or other traditional systems of medicine after a failure of chemotherapy and very aggravated situation most of the time. In Siddha system of medicine, plants, metals and mineral based medicines have been used since ancient times to treat and prevent different types of cancer. Cancer and tumors, whether benign or malignant, are a condition that can be treated with Siddha medicines, either in combination with other treatments or even medicines, as the Siddha medicine does not interfere with any other anti-cancer alternative and can certainly support the patient in a positive way in the healing process. In this regard, effective therapy against cancer, thousands of research on plants and other metallo-mineral preparations from Siddha system of medicine to find potent anti-cancer agents. These medicine have treated many diseases across all over India for thousands of years including cancer. They slow down or block cancer proliferation and stimulate the body's anti-cancer defenses.

In Siddha treatment, there are number of medicines to cure cervical cancer in a non-complicated way. There are also number of literature works to facilitate this. In particular, a potent medicine "*RASAPARPAM*" is mentioned in the classical Siddha book "Aathma Rakshamirtham Ennum Vaithiya Saara Sangiragam" written by Kandhasamy Mudhaliyar.

Mercury

In Siddha medicine, mercury is extensively used in the preparation of medicines after purification. Mercury is also used to in the alchemical and rejuvenation medicine preparations. RASA means elixir of life and the word is attributed to the most important factor responsible for life in various fields of life of knowledge. In the herbal domain it is the essence of a substance. The same meaning is implied to mercury by denoting it as rasa of the mineral kingdom, or in other words the metal of utmost important having distinct properties and unique nature is given the term "Rasa".[6] Even though pure herbal based medicines are effective, mercury based medicines like *Parpam, Mezhugu and Chendhooram* are prepared for the following reasons [7], highly potent, Fast action, Vast utility, Easy to consume, Smaller dose is enough, Acts as rejuvenation medicine Mercury is the most superior among the other metals. Mercury is compared to lord Siva because it has[8,9], Creative power, Protective power, Destructive power.

PARPAM

In Siddha, *parpam* is considered as a higher order of medicine. Even a small dose of *Parpam* can used to cure chronic diseases, complicated diseases and life threatening diseases like cancer etc., The most super natural power present in *Parpam* is its longevity, it has a longevity of 100 years. The peculiarities of *parpam* and its significance are expressed by Theran in his words are as follows.[10]

"Veerathu mikkavai parpangalae……..

……..merukkinai paarappuram"

This Herbo mineral classical Siddha drug *Rasaparpam* is the best drug against cervical cancer. The present study was carried out with the objective of validating the safety and efficacy of *Rasaparpam* and its potent cytotoxic activity in culture of HeLa and SiHa cells.

MATERIALS AND METHODS

Preparation of the Drug

Selection of Drug

"***Rasaparpam***" is one of the Herbo mineral Siddha formulation which was indicated in the Siddha Sastric literature "***Aathmarakchamirtham Ennum Vaithiya Saara Sangiragam"*** written by **Kandhasamy Mudhaliyar.**[11] Hence the present study is taken to validate the scientific background behind this drug through the evaluation of toxicological, pharmacological and elucidation of structural components of the formulation ***Rasaparpam***.

Ingredients

Vaalai Rasam (Purified Elemental Mercury) 35gms.

Gandhagam (Sulphur) 35gms.

Kattuulli – Indian squill (*Urginea indica*) 35gms.

Collection of crude drug

Sulphur, Red sulphide of Mercury and *Plumbago indica* were bought from R. N. Rajan Country raw drug shop at Parrys corner in Chennai, Tamilnadu. The *Kattuulli (Urginea indica*) collected from the Kolli hills, Namakkal District.

Identification and Authentication

The raw materials were identified and authenticated by Botanist and Gunapadam experts, Government Siddha Medical College, Arumbakkam, Chennai. A specimen sample of each raw material has been kept in the department for future reference. (Reg.No:GSMCC/PGGM/0001-0003/14-17).

Purification of raw drug

All the raw materials were purified as per the Siddha literature.[12]

Preparation of the trial drug – *Rasaparpam*

Procedure

Indian squaill and Sulphur – each 35gms were taken and placed in a stone mortar and ground well to get a paste. This is made as a pellet. The pellet was kept in an earthen pot and medicated oil was obtained by calcination method using the equipment – *Kuzhi puda karuvi*. This oil got by *Pudam* (*Kuzhi puda thylam*) was added to *Vaalai Rasam* and kept exposed to

sun light for one day. And the substance was dried. This was ground with the above oil and made as a pellet.

Bricks were taken and crushed into pieces to the size of betel nut. Half of the brick pieces were spread in a round bottom earthen pot. 1 *padi* (1.3lit) of salt was layered above the brick pieces and the pellet was kept over the salt. The pot was covered with earthen dish and sealed with 8 layers of mud pasted cloth and heated using fire woods through high flame (*Kaadakkini*). After that the covering dish was removed. The sublimate was obtained in the upper earthen dish. Finally *Parpam* was collected and ground well. The *Rasaparpam*was collected and kept in an air tight container. The *Rasaparpam* was labelled as RP.

Route of Administration

Oral.

Dosage

Panavedai alavu (488mg).

Adjuvant

Palm jiggery.

Indication

Tumor**, Cervical cancer**, Inguinal bubo, Abscess.

Analytical study as per Siddha literature

Siddhars explained many testing procedures and standardization methods for different kind of medicines. Under this chapter the colour, heaviness, appearance and sense on touch and other test are discussed. For a Siddha formulation these characters are required for standardization.

Standardisation of the Drug

Standardisation of drug means confirmation of its identity, determination of its quality and purity; detection of nature of adulterant by various parameters like morphological, microscopical, physical, chemical and biological evaluations.[13]

Standardization of the drug *Rasaparpam*

Standardization of drugs helps to prove its identity and determination of its quality and potency. Standardization of the Herbo-mineral formulation is based on the qualitative and quantitative analysis through Physico-chemical investigations and instrumental analysis. The Physico-chemical analysis of the prepared Herbo-mineral drug have been done at Central Research Institute, Arumbakkam, Chennai and elemental analysis have been done at IIT, Chennai. (FTIR, SEM, ICP-OES, RAMAN Spectroscopy).

Physico chemical analysis

Physico chemical studies of the trial drug have been done according to the WHO guidelines.

Anti-microbial activity

Availability of microbial load[14]

Enumeration of bacteria by plate count – agar plating technique. The plate count technique was one of the most routinely used procedures because of the enumeration of viable cells by this method.

Sophisticated Instrumental Analysis

To know the test sample RP thoroughly – particle size, quantitative and qualitative values of chemical elements, molecular structure and their functional group, it underwent many analysis done by instruments.

FT-IR (Fourier Transform Infra-Red)

FTIR offers quantitative and qualitative analysis for organic and inorganic samples. Fourier Transform Infrared Spectroscopy (FTIR) identifies chemical bonds in a molecule by producing an infrared absorption spectrum. The spectra produce a profile of the sample, a distinctive molecular fingerprint that can be used to screen and scan samples for many different components. FTIR is an effective analytical instrument for detecting functional groups.[15]

SEM (Scanning electron microscope)

Scanning Electron Microscopy (SEM), also known as SEM analysis or SEM microscopy, is used very effectively in microanalysis and failure analysis of solid inorganic materials. Scanning electron microscopy is performed at high magnifications, generates high-resolution images and precisely measures very small features and objects.[16]

FT-Raman/IR Spectrophotometer[17]

This instrument is used to determine of molecular structure or to indentify materials by infrared spectroscopy and Raman spectroscopy. Generally: Fourier transformation Raman spectroscopy is suitable to investigate chemical composition and structural properties of solid and liquid (polarization measurements) samples. The technique is non-destructive with no sample preparation requirement.

Specifically Dedicated BioRad FT-Raman spectrometer with near-IR excitation laser (1064 nm). The near-IR excitation is a requirement for biological samples allowing non-destructive spectra collection with reduced fluorescence.

The *RASA PARPAM* obtained after sublimation process, shows 6 peaks respectively. The results are shown in Table.No.14.

ICPOES (Inductively Coupled Plasma Optic Emission Spectrometry)

Manufacturer: Perkin Elmer Model.

Optima 5300 DV ICP-OES Inductively Coupled Plasma Spectrometer (ICP) Principle: An aqueous sample is converted to aerosols via a nebulizer. The aerosols are transported to the inductively coupled plasma which is a high temperature zone (8,000– 10,000ºC). The analysts are heated (excited) in different (atomic and/or ionic) states and produce characteristic optical emissions (lights). These releases are separated based on their respective wavelengths and their strengths are measured (spectrometry). The intensities are proportional to the concentrations of analyses in the aqueous sample.

The quantification is an external multipoint linear standardization by comparing the emission intensity of an unknown sample with that of a standard sample. Multi-element calibration standard solutions are prepared from single- and multi element primary standard solutions. With respect to other kinds of analysis where chemical speciation is relevant (such as the concentration of ferrous iron or Ferric Iron), only total essential concentration is analysed by ICP-OES.[18]

TOXICOLOGICAL STUDIES

Acute oral toxicity – OECDguidelines – 423

Acute toxicity study was carried out as per OECD guideline (Organization for Economic Co - operation and Development, Guideline-423.[19]

IAEC No: IAEC/XLVIII/03/CLBMCP/2016, C. L. Baid Metha College of Pharmacy, Thoraipakkam, Chennai.

PHARMACOLOGICAL ACTIVITY

Pharmacological activity in-vitro Anticancer activity determination by MTT assay

HeLa (cervical cancer cells) was initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbeccos modified Eagles medium (Gibco, Invitrogen).

The HeLa cell line was cultured in 25cm 2 tissue culture flask with DMEM supplemented with 10% FBS L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO2 incubator (NBS Eppendorf, Germany).

The viability of cells was evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT (4,5-dimethylthiazol-2-yl) assay method.

Anti-oxidant activity

DPPH assay (2, 2-diphenyl-1-picrylhydrazyl).

The radical scavenging activity of RP extracts was determined by using DPPH assay according to Change tal. (2001).

The decrease in the absorption of the DPPH solution after the addition of an anti-oxidant was measured at 517nm. Ascorbic acid (10mg/ml DMSO) was used as reference.

RESULTS AND DISCUSSION

One of the Siddha Herbo mineral formulation of "*Rasaparpam*" had been exposed to scientific validates. Literary collection, Physicochemical and elemental analysis, toxicological studies and pharmacological studies are done to justify the anticancer activity of *RP* against cervical cancer.

From Review of literature

Discussion on *Gunapadam* review

The poem for general properties of processed quicksilver directly indicates its anti-cancer nature.

As per Siddha classical text, Sulfur by its herbo-mineral formulation indicates anti-cancer property.

The plant *Urginea indica* is used to treat cancer.

Discussion on pharmaceutical review

Parpam

100 years of shelf life denotes its long time efficacy. Being very fine particles in nature, increases the therapeutic effect.

Discussion of pharmacological review

The cell lines for anticancer activity were HeLa and SiHa. They are the genomes of HPV 16 and HPV 18 respectively. These HPV 16 and HPV 18 are responsible for 93% of Cancer cervix.[20]

So, the analysis of pharmacological activity through HeLa and SiHa cell lines are the novel methods for validation which proves the effective anticancer activity of *RP*.

Discussion on materials and methods

The selection of trial drug was taken from the book ***Aathma Rakshamirtham Ennum Vaithiya Saara Sangiragam,*** Written by **Kandhasamy Mudhaliyar**, was approved by the Department of AYUSH as Per Classical Siddha literature.

The ingredients were bought from the authenticated vender and they were identified and authenticated by the experts in Post Graduate Department Gunapadam, GSMC, Chennai. So the ingredients were perfect and original.

The preparation of medicine was done at the well-equipped lab of the Post Graduate Department Gunapadam. So the principles of GMP were adhered during the process.

The analytical parameters were conducted at registered and licensed laboratories only. Thus the result of *Rasaparpam* under various analytical procedures shows the accuracy of RP.

The Siddha Herbo-mineral formulation *Rasaparpam* had been subjected to various studies for its scientific validation and safety assessment. Literary collections, physicochemical and Elemental analysis, Toxicological study, Pharmacological studies are done to prove its efficacy.

Table No.1: Results of Siddha Standardization.

S.No	Parameters	Results of RP	Results of SP
1	Colour	White	White
2	Floating on water	Floats on water	Floats on water
3	Finger print test	Impinged in the furrow of fingers	Impinged in the furrow of the fingers
4	Luster	Lusterless	Lusterless
5	Taste	No specific taste	No specific taste

DISCUSSION

Colour

Rasaparpam is white in colour. The absence of shining denotes that it is free from metals.

Floating on water

Rasaparpam floats on water because of its less specific gravity. It denotes the lightness of the drug, So, it posesses the property of *Parpam*.

Finger print test

Rasaparpam impinged on the lines of the finger. It is denotes the particles are fine and it is in micro size.

Luster

No luster was observed in the *Rasaparpam*. It is due to the change of specific metallic character of raw material after incineration. It denotes that it is well manufactured.

Taste

No specific taste in the *Rasaparpam*. It is due to the change of specific metallic character of raw material after incineration.

Table No. 2: Physical characterization of *Rasaparpam*.

S.No	Parameters	Result
1	Colour	White
2	State of the drug	Powder
3	Consistency	Fine powder
4	Solubility	
	Distilled water	Sparingly soluble
	Ethanol	Sparingly soluble
	Methanol	Sparingly soluble
S.No.	**Parameters**	**Result**
	Propylene Glycol	Not soluble
	Petrolium ether	Not soluble
	Tolune	Not soluble
	Chloroform	Soluble
	Carbon tetra chloride	Soluble
	Xylene	Not soluble
5	Sense on touch	Fine
6	Sense on taste	Tasteless
7	Sense of smell	No significant smell is observed

Physicochemical Analysis

Table No. 3: Results of Physicochemical Analysis.

S.No	Parameters	Result
1	Loss on drying (at 105◦C)	1.0%
2	Total ash	99%
3	Water soluble ash	5.40%
4	Acid insoluble ash	3.65%
5	p^H	6.9

DISCUSSION

Solubility

Solubility is the major factor that controls the bioavailability of a drug substance. It is useful to determine the form of drug and processing of its dosage form. The most frequent causes of low oral bioavailability are attributed to poor solubility and low permeability.[21]

Rasaparpam is soluble in major solvents and sparingly soluble in some solvents. This proves its efficiency of solubility in the stomach indirectly, increasing the bio availability.

P^H value

Rasaparpam shows acidic P^H.

The P^H level plays a role in enzyme activity by maintaining the internal environment thus regulating the homeostasis. It is also an important factor for drug absorption.[22] Because of

the acidic nature, the drug is more readily absorbed in an acidic medium like stomach which enhances the bioavailability of the drug.

Loss on drying

Loss on drying (LOD) gives the total amount of volatile content and moisture (water) present in the drug. The stability of a drug and its shelf-life are dependent on moisture content. Moisture increase can adversely affect the active ingredient.

Low moisture content- drug could get maximum stability and better shelf life. Since the drug has low loss on drying, the moisture content is less which is suitable for medicine preparation.

Ash values (Total Ash value)

Low total Ash value indicates the trial drug contains plant organic derivatives. It is not subjected to calcination process.

Acid insoluble ash

Lower the acid insoluble value better will be the drug quality[23]. The drug ensures a low value of acid insoluble ash indicating that the preparation did not contain any sand, dust and stones.

Water soluble ash

Decreased water soluble ash value (5.40 %) indicates easy facilitation of diffusion and osmosis mechanisms.

Biochemical Analysis

Table No. 4: Results of Basic radicals.

S.No	Parameters	Result
1	Test for Potassium	Negative
2	Test for Calcium	Positive
3	Test for Magnesium	Positive
4	Test for Ammonium	Negative
5	Test for Sodium	Negative
6	Test for Iron (ferrous)	Negative
7	Test for Zinc	Positive
8	Test for Aluminium	Positive
9	Test for Lead	Negative
10	Test for Copper	Negative
11	Test for Mercury	Positive
12	Test for Arsenic	Negative

The biochemical analysis for basic radicals of *RP* shows the presence of Calcium, Magnesium, Zinc, Aluminium and Mercury.

Table No. 5: Results of Acid Radicals.

S.No	Parameters	Result
1	Test for Sulphate	Positive
2	Test for Chloride	positive
3	Test for Phospate	Negative
4	Test for Carbonate	Negative
S.No	**Parameters**	**Result**
5	Test for Fluoride & Oxalate	Negative
6	Test for Nitrate	Negative

The bio chemical analysis for acid radical of *RP* shows the presence of Sulphate and Chloride.

DISCUSSION

The presence of these radicals helps *RP f*or its therapeutic effect.

Zinc is needed for immune function, wound healing and blood clotting.

Mercury helps to destroy the cancer cells and reduces the tumor growth.

Mercuric chloride has cyto toxic effects.

Sulphate contains anti-cancer property.

Anti- microbial load

Availability of bacterial and fungal load in Rasaparpam

Table No. 6: Bacterial and Fungal dilution.

Micobes	Dilution	Result
Bacteria	10^{-4}	7
Bacteria	10^{-6}	6
Fungi	10^{-3}	Nil
Fungi	10^{-2}	Nil

DISCUSSION

This is one of the Siddha herbo mineral formulations which are prepared by using plant materials that are prone to contamination. The contamination of herbal drugs by micro-organism not only cause bio deterioration but also reduces the efficacy of drugs.

The toxic effect produced by microbes makes the herbal drugs to give no response for human consumption because the contaminated drug may develop unwanted disease instead of disease being cured.

FTIR (Fourier transform infrared spectroscopy)

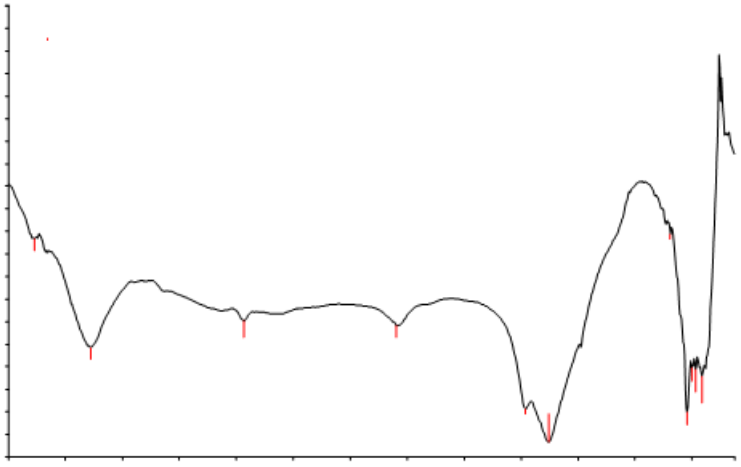


Fig.No.1.

Table 7: FTIR- results of RP.

Frequency, cm^{-1}	Bond	Functional Group
3425	O-H Stretch, H-bonded	Alcohol, Phenols
1638	N-H bend	1° amines
1184	C-H wag (-CH_2X)	Alkyl halides
1103	C-O Stretch	Carboxylic acids, esters, ether
676	C-H "oop"	Aromatics
618	-C=C-H:C-H bend	Alkynes
602	C-Br stretch	Alkyl halides
587	C-Cl stretch	Alkyl halides

DISCUSSION

The wave numbers from 4000cm-1 to 1500cm-1 gives details for identification of functional group. The wave number from 1500cm -1 to 400 cm-1 provides particulars about molecular fingerprint. The above result showed the presence of functional group like Phenol, Alcohols, Alkynes, Amines, Carboxylic acids, Esters, Ether and Alkyl halides in *Rasaparpam*. They may be responsible for the presence of anticancer action of *RP* in cervical cancer.

Alcohols

OH group of *RP* has higherr potential towards inhibitory activity against microorganism.

Phenols

Phenols of *RP* possess highly Anti-Oxidant property which enchances its effect against the disease.

Carboxylic Acid

Benzene-poly-carboxylic Acid Complex (BP-CI) is a novel anticancer complex against human cancer cells.

Ether

Certain ether lipids such as 1-0-octadecyl-2-0 methyl-rec-glycero-3-phosphocholine represent a new class of anti -neoplastic agents. These ether lipids have been shown to be cytotoxic for a wide variety of tumors.

Alkyl halides

High propotion of low molecular weight alkyl halides may be weakly carcinogenic and provide evidence supporting and eletrophillic hypothesis of carcinogenesis.[24]

SEM (Scanning electron microscope)

The following image is done by 80000X via 500nm aperture shows maximum depth focused.

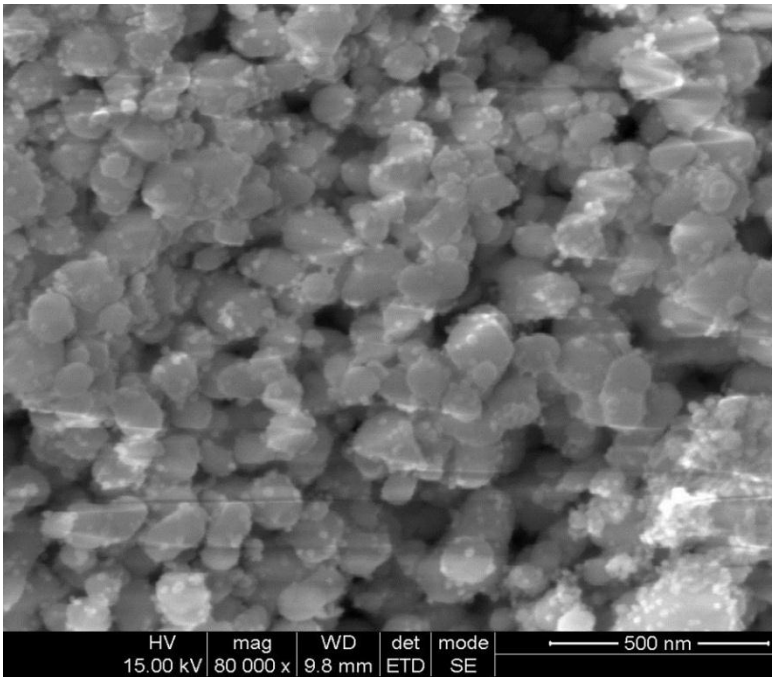


Fig.No.2: Showing nano particles in SEM image of 500nm.

The particle size ranging from 80nm-120nm.

Discussion on SEM reports

Nanoparticles, according to the American Society for Testing and Materials (ASTM) standard definition, are particles with lengths that range from 1 to 100 nm in two or three dimensions.

The test drug *Rasaparpam* contains nano particle. Nano particles present in the drug results in a better bioavailability and facilitates absorption.

Nanotechnology a promising way from cancer management towards cancer elimination.

The particles of nano size show that the drug may easily enter the cells at the molecular level to treat the disease rapidly and increase the therapeutic effect.

ICP-OES Results and Discussion

Table No. 8: ICP-OES Results of *RP*.

S.No	Elements	Detected levels
1	Aluminium	BDL
2	Arsenic	BDL
3	Cadmium	BDL
4	Copper	BDL
5	Mercury	13.879mg/L
6	Potassium	03.821 mg/L
7	Sodium	04.300 mg/L
8	Nickel	BDL
9	Lead	BDL
10	Sulfur	70.304 mg/L

DISCUSSION

From the above results, the heavy metals Arsenic, Cadmium and Lead were found below detection level. Mercury, Sodium and Potassium are observed within the permissible limits. Hence the safety of the drug *Rasaparpam* is ensured.

Table No. 9: Raman Spectroscope – results of RP.

Frequency, cm^{-1}	Functional group	Raman Bond
3616.09	O-H	Weak
3097.39	=C-H	Strong
2200.71	C=C	Strong
940.01	C-O-C	Medium
814.89	C-O-C	Medium
371.57	(CC) Aliphatic chains	Strong

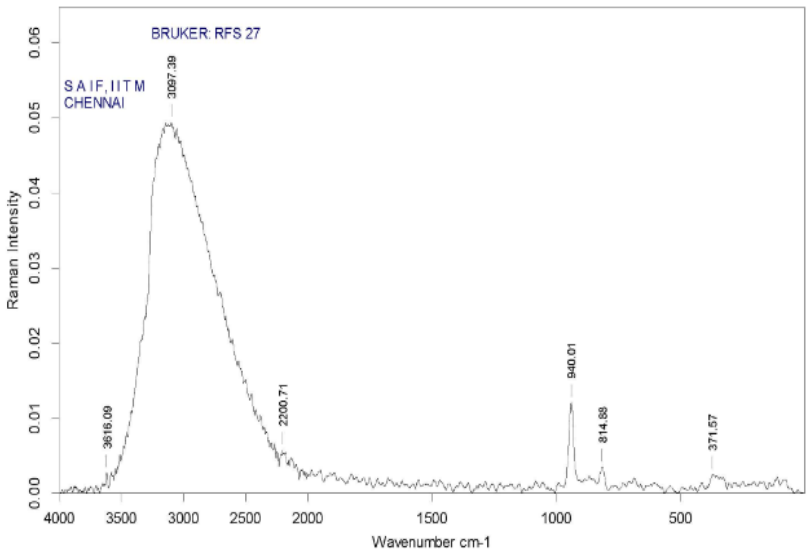


Fig. No: 3. Raman spectrometer peaks of RP.

DISCUSSION

The drug obtained after sublimation process as per standard Siddha- SOP shows carbon linkages with Hydrogen, Carbon, Oxygen and aliphatic chains.

These bond natures might be Ionic, Covalant, coordinate covalent (C-O-C bond linkage, C=C bond linkage).

The sublimation process convert the heterogeneous material into homogeneous substance and creating new complex compounds of various bonds.

Electronegativity is a measure of the tendency of an atom to attract a bonding pair of electrons.

The bonding pair of electrons of one atom is pulled towards the other end of the atom bond.

The larger the difference in electro negativity between the two atoms involved in the bond, makes it more ionic.

This electronegativity/nucleophillic property of the *Rasa parpam* makes free radicals to get attracted by this electron clouds and some of the new bonding make hold the metabolites from damaging the cells and detoxify them and also these characters favours the phenomenon of electron transport chain and maintain the cellular metabolism properly and ensures the Anti-Oxidant property.

Essential properties of anti-oxidants: Oxidative stress induced cell damage through damage to proteins, lipids and DNA. It may also alter signaling pathways redox sensitive to changes involved in the response of apoptosis. The antioxidants are currently the subject of many studies because, in addition to some interest in the preservation of comestibles, they could be useful in the prophylaxis and treatment of diseases in which oxidative stress is implicated.

From the present study, it was concluded that the *Rasaparpam* extract has good anti-oxidant activity at higher concentrations.

So, the *Rasaparpam* due to its anti-oxidant property could eliminate cancer cells.

ACUTE ORAL TOXICITY

Dose finding experiment and its behavioral signs of Toxicity for *Rasaparpam.*

Observation done

Table No. 10: Observational study.

S.No	Group	Day
1	Body weight	Slightly decreased
2	Assesments of posture	Normal
3	Signs of convulsion Limb paralysis	No signs of convulsion and paralysis
4	Body tone	Normal
5	Lacrimation	Slightly increased
6	Salivation	Normal
7	Change in skin colour	Normal
8	Piloerection	Abnormal
9	Defection	Normal
10	Sensitivity response	Normal
11	Locomotion	Normal
12	Muscle gripe	Normal
13	Rearing	Normal
14	Urinatiom	Normal

Table No. 11: Observational study Results.

Dose mg/kg	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
RP 200 mg/kg	+	-	+	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-

1. Alertness 2. Aggressiveness 3. Pile erection 4. Grooming 5. Gripping 6. Touch Response 7. Decreased Motor Activity 8. Tremors 9. Convulsions 10. Muscle Spasm 11. Catatonia 12. Muscle relaxant 13. Hypnosis 14. Analgesia 15. Lacrimation 16. Exophthalmos 17. Diarrhea 18. Writhing 19. Respiration 20. Mortality.

(+ Present, - Absent)

Table No. 12: Body weight observation.

DOSE	DAYS		
	1	7	14
CONTROL	180.6±1.44	181.4 ± 4.32	183.2 ± 7.63
HIGH DOSE	190.5± 7.75	188.7 ± 1.67 ^{ns}	184.4 ± 2.67^{ns}

Acute toxicity Discussion

In the acute toxicity study, the rats were treated with different concentration of *Rasa Parpam* from the range of 5mg/kg to 200mg/kg.

This dose level did not produce signs of toxicity, behavioral changes and mortality in the test groups as compared to the controls when observed during 14 days of the acute toxicity experimental period.

These results showed that a single oral dose of the extract showed no mortality of these rats even under higher dosage levels indicating the high margin of safety of this extract.

In acute toxicity test *Rasaparpam* was found to be non-toxic at the dose level of 200mg/ kg body weight.

28 days Repeated Oral Toxicity of Rasaparpam

Table No. 13: Body weight of wistar albino rats group exposed to *RP*.

DOSE	DAYS				
	1	7	14	21	28
CONTROL	180.6±3.62	181.4 ± 4.14	183.7 ± 9.61	184.6 ± 3.03	185.7 ± 1.31
LOW DOSE	183.2 ± 1.14	181.4 ± 2.12	179.6±2.36	177.2 ± 4.78*	174.12± 2.39**
MID DOSE	186.6± 1.64	181.3 ± 2.74	179.4 ± 8.32	174.1 ± 3.16*	172.7 ± 3.82**
HIGH DOSE	184.4± 6.74	179.7 ± 3.64	176.4 ± 1.51	170.1 ± 4.66*	164.4± 3.76**

NS- Not Significant, ** ($p > 0.01$), * ($p > 0.05$), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test).

Table No. 14: Haematological parameters of Wistar albino rats group exposed to *RP*.

Category	Control	Low dose	Mid dose	High dose
Haemoglobin(g/dl)	13.8±0.88	13.90±1.16	11.14±0.66	9.28±1.16*
Total WBC ($\times 10^3$ l)	11.91±0.59	11.85±1.23	10.08±1.21	8.110±2.27*
Neutrophils(%)	33.65±0.06	33.3±1.24	32.11±2.16	30.20±1.10
lymphocyte (%)	70.24±1.48	70.02±1.12	69.20±1.16	60±1.26*
Monocyte (%)	0.86±0.07	0.85±0.19	0.72±0.13	0.71±0.60
Eosinophil(%)	0.54±0.09	0.54±0.12	0.62±0.16	0.72±0.04
Platelets cells10^3/µl	687.17±8.76	678.71±9.16	623.18±2.20	627.16±3.74
Total RBC 10^6/µl	7.99±0.12	7.79±1.57	7.62±0.19	6.05±0.12*
PCV%	37.79±0.6	37.35±1.23	32.98±1.18	25.82±2.14*
MCHC g/dL	33.6±2.23	33.29±1.19	30.18±1.12	34.03±1.14
MCV fL(µm^3)	49.07±3.64	47.28±8.12	45.20±1.24	4.22±1.94

N.S- Not Significant, ** ($p > 0.01$), *($p > 0.05$), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test).

Table No. 15: Biochemical Parameters of Wistar albino rats group exposed to *Rasa Parpam.*

BIOCHEMICAL PARAMETERS	CONTROL	LOW DOSE	MID DOSE	HIGH DOSE
GLUCOSE (R) (mg/dl)	74.45±13.4	78.16±1.24	92.26±1.22	110.12±9.60
T.CHOLOSTEROL(mg/dl)	115.26±1.83	118.45±1.13	132.42±1.78	156.22±1.93
TRIGLY(mg/dl)	46.35±1.48	48.22±1.28	49.58±1.80	59.66±1.13*
LDL	73.8±2.43	75.24±3.14	82.14±1.24	96.64±4.12*
VLDL	15.2±2.44	15.82±1.14	18.44±2.14	19.24±4.16
HDL	26.66±6.88	26.16±1.24	24.68±2.16	20.78±1.12*
Albumin(g/dL)	3.3±0.17	3.23±0.22	2.48±2.02	2.14±3.16*

NS- Not Significant, ** (p > 0.01), * (p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test).

Table No. 16: Renal function test of Wistar albino rats group exposed to *Rasa Parpam.*

PARAMETERS	CONTROL	LOW DOSE	MID DOSE	HIGH DOSE
UREA (mg/dl)	13.35±0.99	14.81±1.26	16.26±1.18	21.28±3.12*
CREATININE(mg/dl)	0.58±0.08	0.48±0.06	0.72±0.14	0.94±0.12*
BUN(mg/dL)	15.12±0.10	15.12±0.28	16.28±0.14	16.90±1.22
URIC ACID(mg/dl)	5.37±0.35	5.11±0.43	6.72±2.15	7.28±0.14*

NS- Not Significant, ** (p > 0.01), * (p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test).

Table No. 17: Liver Function Test of Wistar albino rats group exposed to *Rasa Parpam.*

PARAMETERS	CONTROL	LOW DOSE	MID DOSE	HIGH DOSE
T BILIRUBIN(mg/dl).	0.50±0.07	0.58±0.16	0.62±0.18	0.76±0.15*
SGOT/AST(U/L)	114.95±1.39	118.15±2.11	131.21±1.23	145.55±1.23*
SGPT/ALT(U/L)	71.23±1.28	76.91±1.59	82.34±2.18*	86.32±1.28*
ALP(U/L)	146.25±8.77	144.2±6.27	149.16±4.17*	153.3±4.25*
T.PROTEIN(g/dL)	6.32±0.38	6.12±1.34	5.76±0.23*	5.10±1.26*

NS- Not Significant, ** (p > 0.01), * (p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test).

DISCUSSION

The dose selected for the sub acute toxicity study was 20mg, 40mg/kg of *Rasa parpam.*

All the animals were free of intoxicating signs throughout the dosing period of 28 days.

No physical changes were observed throughout the dosing period.

Mild toxic but no mortality was observed during the whole experiment. No abnormal deviations were observed.

No significant changes were observed in the values of different parameters studied when compared with controls and values obtained were within normal biological and laboratory limits.

The weights of organs recorded that shows mild differences in the treatment when compared to control group. This indicates that *Namachivaya Chendooram* induce mild changes in liver and kidney but not toxic to rest of the organs.

There was slight changes were observed in hemoglobin (Hb), red blood cell (RBC). No significant changes in white blood cell (WBC), packed cell volume (PCV), Erythrocyte sedimentation rate (ESR) in all the treated groups as compared to respective control groups.

Histopathology Examination

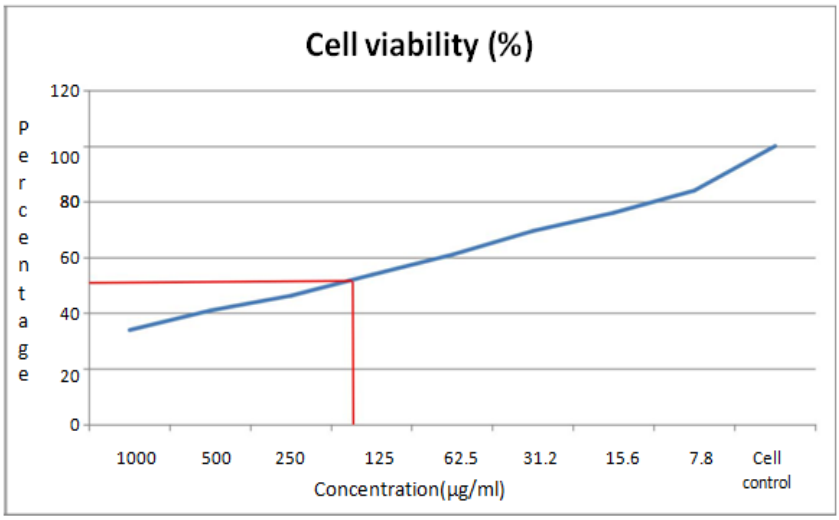
Histopathology studies were carried out on liver, kidney and spleen were recorded. Blood samples for haemotological and bio chemical analysis were taken from common carotid artery.

All tissues were preserved in 10% neutral buffered fomaldehyde solution for histopathological examination. The internal organs and tissues were observed for gross lesions.

PHARMACOLOGICAL STUDY

Table No. 18: Anticancer effect of *Rasaparpam on HeLa* cell line.

S.No.	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	Neat	0.495	34.13
2	500	1:1	0.595	41.03
3	250	1:2	0.672	46.34
4	125	1:4	0.781	**53.86**
5	62.5	1:8	0.887	61.17
6	31.2	1:16	1.008	69.51
7	15.6	1:32	1.105	76.20
8	7.8	1:64	1.219	84.06
9	Cell control	-	1.450	100



Graph 1.

Graph-1 shows the drug dose and % of Inhibition of HeLa cells after the *Rasa parpam* extract treatment. It can be observed by the result of MTT assay that the IC dose of *Rasa parpam* is 125µg/ml. As the dose increases the HeLa cell viability decreases. It was found that the % growth inhibition increasing with increasing concentration of *Rasaparpam* steadily up to 7.8 µg/ml on *HeLa* cell line (Table No: (23) and Graph(1) and that IC value on *HeLa* cell line was 50 and R value was 1.450.

DISCUSSION

Cytotoxic effect by MTT assay

MTT ia a yellow water soluble tetrazolium salt. Succinate dehydrogenase, a mitochondrial enzyme in living cells, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells.

This assay is based on the metabolic reduction of 3- (4, 5-dimethylthiazol-2-yl) -2, 5-difeniltetrazol (MTT) by mitochondrial enzyme succinate dehydrogenase in a coloured compound blue (formazan), allowing to determine the functionality of the mitochondrial treated cells. This method has been widely used to measure survival and cell proliferation.

The amount of living cells is proportional to the amount of formazan produced. Cell lines derived from NCCS, Pune were free from any kind of bacterial and fungal contamination.

To determine the cytotoxic effect of novel Siddha formulation *Rasa parpam* against HeLa cells. The experiment was screened at different concentrations to determine the IC_{50} using MTT assay. A chart was plotted using the % cell vibility in Y-axis and concentration of the test sample in X-axis.

The percentage of growth inhibition was found to be increasing with increasing concentrations of test drug. The IC_{50} of test sample in HeLa cell line was found to be 125µg/ml. This confirms that the Siddha formulation *Rasaparpam* has promising anti-cancerous effect.

RASA PARPAM at different doses (7.8-1000µg/ml of 5% MEM) was administered for 24 hrs. It was found that the number of cells decreases as the dose increases and at approximately 125 µg/ml dose of extract, 50% of the cells (HeLa cells) were less as compared to normal control as shown in Fig.No:23.

The percentage of cells viability was determined by calculating the O.D of treated against the control.

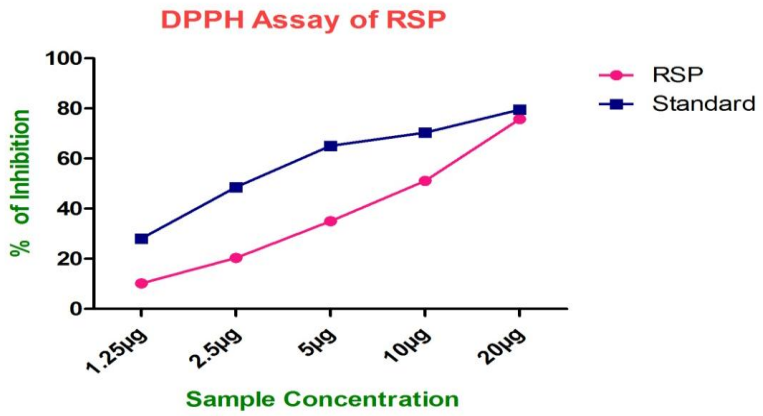
Reading optical density (OD) is performed in a spectrophotometer at a wavelength of 570nm. Comparison values are made on a basis of 50% inhibition of growth (IC_{50}) in treated cells with specific agents.

Anti-Oxidant activity Activity

Table No. 19: DPPH Assay of *Rasaparpam*.

Concentration (µg/ml)	Absorbance		Percentage of Inhibition	
Rasaparpam	Drug	Standard	Drug	Standard
Control	0.5432	0.327	-	-
1.25	0.4876	0.235	10.23	28.14
2.50	0.4327	0.168	20.34	48.63
5.00	0.3527	0.114	35.07	65.14**
10	0.2653	0.097	51.16*	70.34
20	0.1317	0.067	75.75	79.52

(µg/ml) microgram per mililiter. Drug: RP (1.25µg/ml-20µg/ml) Standard: Ascarbic acid (10mg/mlDMSO).



Graph-2

DPPH stable free radical method is an easy, rapid and sensitive way to survey the antioxidant activity of *Rasaparpam* extract. The antioxidant molecules can quench DPPH free radicals by providing hydrogen atom or by electron donation and a colorless stable molecule 1, 1 diphenyl-2-picryl hydrazyl is formed and as a result to which the absorbance at 517 nm of the solution is decreased.

In the present study the *Rasaparpam* extract was analyzed was able to decolorize DPPH and the free radical scavenging activit y was expressed as the percentage decrease in absorbance.

(10mg/mlDMSO) have a concentration-dependent anti-radical activity which was tabulated in Table No.

A maximum of 75.75% and 79.52% anti-radical effects are exercised by *Rasaparpam* and standard drug ascorbic acid at concentrations of 20µg/ml respectively. Minimum percentage of inhibition 10.23% and 28.14% anti-radical effects are manifested by *Rasaparpam* and standard drug ascorbic acid at concentrations at 1.25µg/ml.

This indicated that % of inhibition increased with increase in concentration of both the standard and *Rasaparpam* extract. But the *Rasaparpam* extract has lower DPPH scavenging activity than that of standard. From the present study, it was concluded that the *Rasaparpam* extract has good anti-oxidant activity at higher concentrations.

It is known that oxidative stress induced cell damage not only through damage to proteins, lipids and DNA. It may also alter signaling pathways redox sensitive to changes involved in the response of apoptosis. The antioxidants are currently the subject of many studies because,

in addition to some interest in the preservation of comestibles, they could be useful in the prophylaxis and treatment of diseases in which oxidative stress is implicated. Many studies realized on natural products have proven that they are especially phenolic compounds who are responsible for their antioxidant activity.

Several studies have shown the link between the traditional drug formulations rich in anti-oxidants and the incidence certain diseases such as **cancer**, diabetes, heart disease and other diseases related to aging. Phenolic compounds could prevent cancer by the action antioxidant and the modulation of several functions of proteins. Phenolic compounds can prevent carcinogenesis by affecting the molecular events in the triggering, promotion and progression stages.

Here, the reactive oxygen species (ROS) may be the triggers apoptotic process. In recent years numerous properties have been described about these compounds such as the ability to inhibit cell cycle, proliferation cellular and oxidative stress and induce detoxification enzymes, apoptosis and stimulate the immune system. It is therefore hypothesized that *Rasaparpam* of its anti-oxidant power could eliminate cancer cells.

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